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14. ABSTRACT
We tested the hypothesis that hypoxic breast cancer cells would be more permissive to herpes simplex (HSV-1)-derived plasmids. Since hypoxic cells are more resistant to traditional therapies, this work could provide a new strategy for treating aggressive breast cancer. The general hypothesis was that ERK (extracellular receptor kinase) would be active in hypoxic cells rendering them susceptible to oncolytic viruses, such as R3616. R3616 lacks the γ1 34.5 gene that normally blocks the host cell's attempt to inhibit viral protein synthesis. We determined whether hypoxic MCF-7, MDA-MB-231, andMDA-MB-435 cells would be more permissive to R3616. We observed that hypoxic MDA-MB-231cells were indeed more permissive to R3616; however, MDA-MB-435 cells were not. This observation may lead to future experiments to identify novel treatment modalities for hypoxic cells. However, considering that ERK1/2 activation is constitutive in MDA-MB-231 cells, it is possible that other hypoxic signaling pathways are important in rendering the MDA-MB-231 cells permissive to the oncolytic virus.

15. SUBJECT TERMS
Oncolytic virus, hypoxia, breast cancer cells, treatment modalities

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INTRODUCTION: The basis for this project, **selective oncolytic treatment for hypoxic breast cancer cells**, is the idea that herpes simplex (HSV-1)-derived oncolytic viruses, such as R3616, require ERK (Extra-cellular receptor kinase) activation, to be infective in tumor cells. These viruses, such as G207 or R3616, lack the viral ribonucleotide reductase gene ICP6 and the viral gene γ_1 34.5 (Fu and Zhang, 2002), and are safe for cancer therapy since they cannot replicate in non-transformed cells. The γ_1 34.5 prevents host cells from shutting down protein synthesis by RNA-dependent protein kinase (PKR) by reversing PKR-mediated eIF-2a phosphorylation. HSV-1 oncolytic viruses can replicate in a subset of tumor cells that over-activate the MEK (MAP kinase kinase) pathways (Smith et al., 2006). The modified virus R3616 can replicate in those tumor cells that over-stimulate MEK, which blocks eIF2-2a phosphorylation and downregulates apoptotic signals (Smith et al., 2006). Since hypoxia has been observed to activate ERK, we hypothesized that hypoxic cells would be more permissive to R3616. This is of particular importance for cancer treatment, since hypoxic breast cancer cells are refractory to treatment by radiation and particular chemotherapeutic agents. The tasks of this project are thus to establish breast cancer cell lines (task 1), measure ERK activation (task 2), determine whether HSV-1 and R3616 would kill MCF-7 and other breast cancer cells under different O2 concentrations (task 3 and 4), determine whether MEK inhibitors would confer resistance to oncolytic virus infection (Task 5), and measure infectivity in breast cancer cell lines that have a Ki-RAS mutation (task 6). We thus aimed to test the hypothesis that since Herpes simplex (HSV1) **oncolytic viruses require MEK activation** for replication, and **MEK activation is enhanced by hypoxia**, the HSV-1 oncolytic virus may preferentially replicate and kill hypoxic cancer cells, thus rendering them amenable to treatment.

BODY: The body of our report is divided into the six tasks as described by the original Statement of Work (SOW).

Task1. Establish cell cultures of MCF-7, MDA-MB-435, MDA-MB-231, and SKBR-3. Cell lines not available will be purchased from the American Type Culture Collection (ATCC) (Months 1-3).

Progress: We established cell lines of MCF-7, MDA-MB-231, and MDA-MB-435 in the laboratory. These cell lines were purchased from the American Type Culture Collection. However, we did not establish SKBR-3 cell lines. The cell lines were selected because they have different p53 and Ras mutations, and are ER+ or negative. For example, MCF-7 cells are p53 positive and do not contain a Ras mutation, while MDA-MB-231 cells are ER- p53- and do contain a Ras mutation. MDA-MB-435 also contains a Ras mutation. In addition, MDA-MB-231 cells are derived form a more aggressive breast cancer cell line.

Task2. Measure ERK1/2 activity in cell lines under normoxic and hypoxic conditions (1% or 5% O_2). ERK activity will be measured by measuring phosphorylated forms of p42 (ERK1) and p44 (ERK2) on western blots (Months 2-4).

a. MCF-7 (Months 2-3).
b. MDA-MB-435, MDA-MB-231, and SKBR-3 (Months 2-4).

Progress: We had hypothesized that since ERK activation of the Ras pathway correlates with mutant R3616 activation, and hypoxia activates ERK, we should detect more ERK activation in MCF-7 cells after cells were exposed to hypoxic conditions at 1% or 5%. We measured ERK activity in MCF-7 cells under normoxic and hypoxic conditions by Western blots using a monoclonal antibody against phosphorylated ERK1/2, using the protocol described in Davis et al. (2006). We found that in order to culture MCF-7 cells in 2% O2 we needed to use low serum concentrations (0.25%) and media that did not contain phenol red. We obtained both cytosolic and nuclear extracts. However, in two independent experiments we did not detect ERK activation after MCF-7 cells were exposed to hypoxia. We are now collaborating with R. Buttyan's group to determine whether we can enhance the detection of activated ERK under hypoxic conditions.

Task 3. Determine whether the oncolytic virus HSV-1 would kill MCF-7 cells under varying hypoxic conditions (1%, 3%, 5% O_2). Establish m.o.i. with maximum killing (Months 4-7).

a. R3616
b. Other oncolytic HSV-1 oncolytic viruses, including G207 and Fu-10 (Months 5-6).

Progress: We found that hypoxic MCF-7 cells were only slightly more permissive than normoxic MCF-7 cells to normoxia. We repeated experiments that were done by Smith et al., and found that essentially the same number of plaque forming units consistent with their report. These experiments were done under normoxic conditions for periods of 24-72 hrs.

However, under hypoxic conditions we found it difficult to maintain MCF-7 cells for more than 24 hrs. The media became increasingly acidic, due to the increased glycolysis. Although we did used buffered media with 10 mM Hepes, we did not completely resolve the problem. However, we did find that cells maintained in low serum could be maintained until 48 hrs (Figure 1).

Task 4. Determine whether the oncolytic virus R3616 would kill MDA-MB-435, MDA-MB-231, and SKBR-3 cells under varying hypoxic conditions (1%, 3%, 5% O_2). Titer virus in green monkey kidney (VERO) cells. Establish multiplicity of infection (m.o.i.) with maximum killing (Months 7-10).

Progress: We detected killing of MDA-MB-231, and MDA-MB-435 cells after exposure to R3616 virus. We found a maximum killing of MDA-MB-231 cells with a m.o.i of 0.1. Interestingly, we found that the hypoxic MDA-MB-231 cells were the most permissive to the R3616 virus, while normoxic MDA-MB-435 cells actually more permissive than hypoxic cells. We found that normoxic MDA-MB-435 were two orders magnitude more permissive than hypoxic MDA-MB-435 (14 x 10e6 pfu) after a 48-hour infection (n=2). We found that hypoxic MDA-MB-231 cells exposed to either 1% O2 or 5% O2 were significantly more permissive (3-9 fold) to R3616 than normoxic cells (Figure 1). We present an example of a titer dish from normoxic and hypoxic cells (Figure 2).

We found that a significant fraction of cells were infected with virus at MOI of 1 or greater. We used immunofluorescence to detect GP6 (coat protein) that is indicative of the late stages of infection after 6 and 24 hrs. We also used HSC70 (heat shock chaperone) and ICP27 to detect the early stages of infection. The results indicated that there was a significant accumulation of GP6 in hypoxic MDA-MB231 cells (Figure 3).

Task 5. Determine whether specific inhibitors of MEK, such as PD98058 and PD98059, confer resistance to R3616 in MCF-7 cells under hypoxic conditions (Months 8 –10).

Progress: We have purchased the MEK inhibitors but did not complete the task.

Task 6. Determine whether hypoxic conditions that upregulate MEK, lead to R3616 sensitivity in other breast cancer cell lines, including MDA-MB-435 and MDA-MB-231, which harbor a Ki-RAS mutation, and SKBR-3 (Months 10-12).

Progress: We did not determine whether hypoxic conditions that can upregulate MEK would also lead to R3616 sensitivity.

KEY RESEARCH ACCOMPLISHMENTS:

- Both normoxic and hypoxic MDA-MB-231 and MDA-MB-435 cells are permissive to the HSV1 derivative R3616

- Hypoxic MDA-MB231 cells are significantly more permissive to R3616 than normoxic cells. This is significant because it informs us that hypoxic breast cancer cells may indeed be susceptible to R3616 viruses.

REPORTABLE OUTCOMES: Publication pending:

Hypoxic breast cancer MDA-MB-231 cells are more permissive to the HSV-1 derived oncolytic viruses

CONCLUSIONS: In summary, we have made significant progress in establishing breast cancer cell lines, measuring ERK activity, and determining viral infection for the MCF-7 and MDA-MB231 cell lines (tasks 1, 2, 3, 4). Interestingly, we have found that hypoxia significantly enhances R3616 infectivity in MDA-MB231 cells, less so for MCF-7 cells, but not in MDA-MB-435 cells. We have determined that M.O.I of 0.01 yields the best results (task 3). Our data do not support the notion that hypoxic cells are more permissive due to an increase in ERK activation. Indeed ERK appears constitutive in all the cell lines tested, and was previously shown to be constitutively active in MDA-MB-231 cells. We have not excluded the possibility that Ras mutations may confer enhance sensitivity, and this is still being investigated. Although the MEK inhibitors have been purchased, we have yet to determine whether specific inhibitors of MEK confer resistance to oncolytic virus in either MCF-7 or MDA-MB231 cells (task 5). Thus, other hypoxic signaling pathways may be important in conferring increase susceptibility to R3616, and these will be investigated in future studies. Thus, understanding which breast cancer cells are permissive oncolytic viruses may enable clinicians to design better therapeutic modalities for aggressive breast cancer cells.

REFERENCES:

Davis F., Tang HY, Shih A, Keating T., Lansing L, Hercbergs A., Fenstermaker R, Mousa S, Davis P, Hung-yun L. Acting via cell surface receptor, thyroid hormone is a growth factor for glioma cells. Cancer Res. 2006, 66:7270-7275

Fu, X and Zhang, X. Potent systemic antitumor activity from an oncolytic herpes simplex virus of syncytial phenotype. Cancer Res. 2002 62: 2306-2312

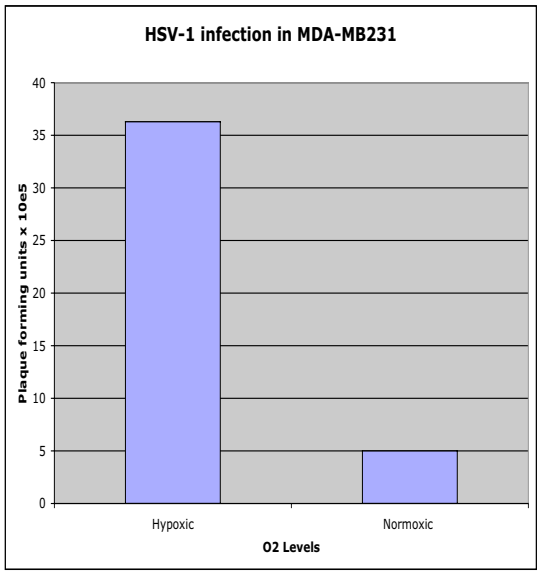
Smith, K., Mezhir, J., Bickenback, K., Veerapong, J., Charron, J., Posner, M., Roizman, B., and Weichselbaum, R. Acitvated MEK suppresses activation of PKR and enables efficient replication and in vivo oncolysis by D g_1 34.5 mutants of herpes simplex virus I. J. Virol., 2006 80: 1110-1120

APPENDICES: Figures 1,2,3 showing supporting data

SUPPORTING DATA; (Subsequent Pages)

Figure 1

A



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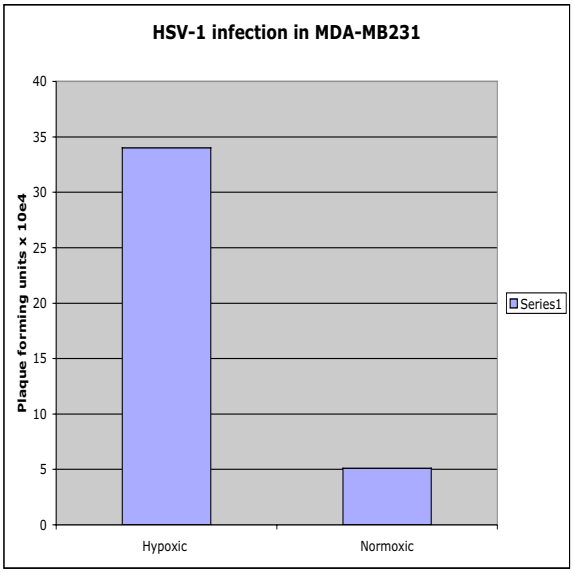
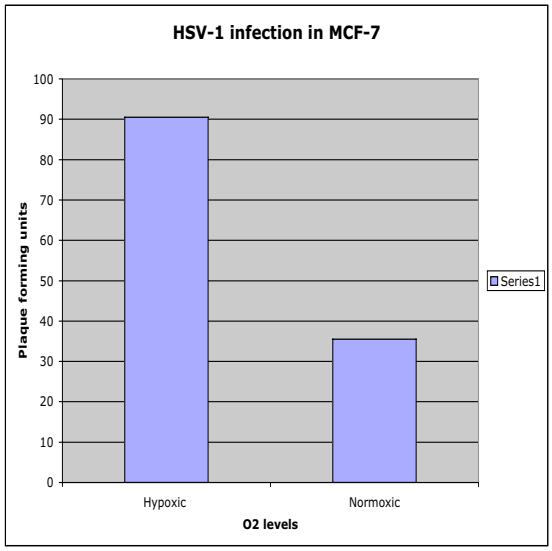
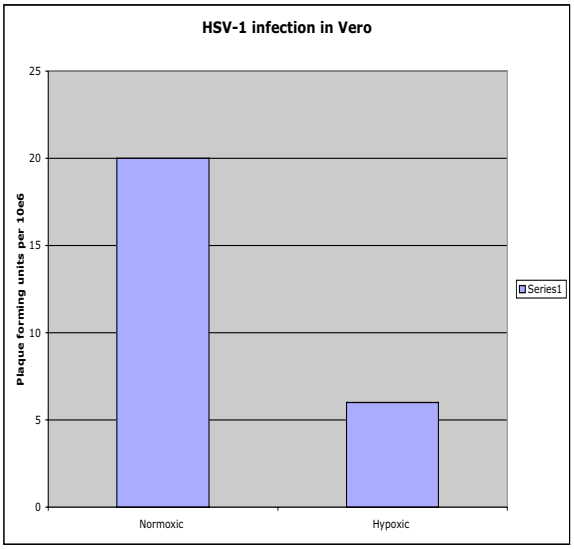






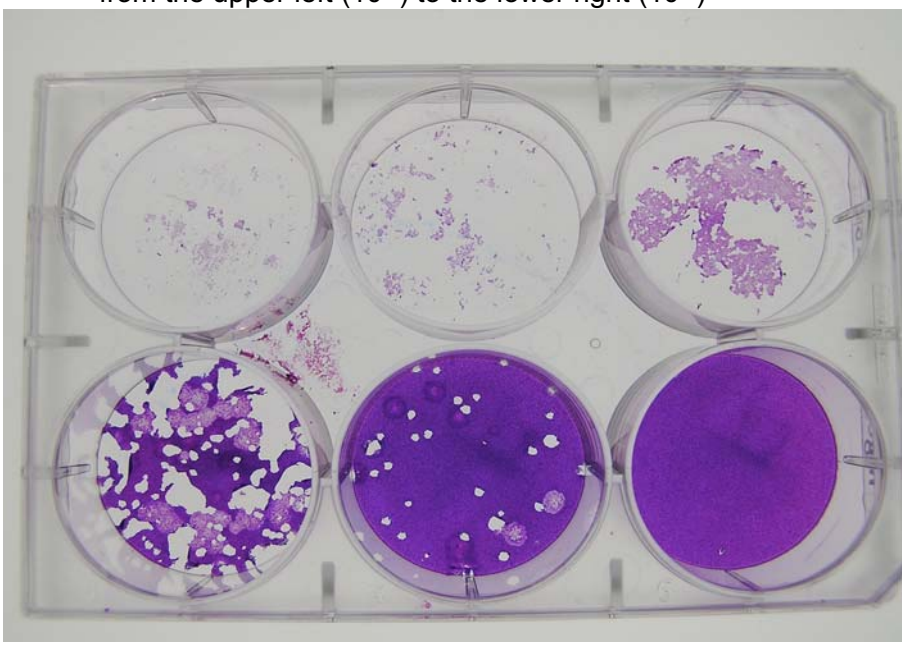
C

D

Figure 1: Infection of the modified HSV-1 virus, R3616, in MDA-MB231, MCF-7, and Vero cells. Cells were infected with R3616 as described by Smith et al., 2006, and samples collected after 48 hours. Plaque forming units were determined by standard plaque assays. M.O.I = 0.01 in all assays. A), Infection of R3616 in normoxic and hypoxic (2% O2) MDA-MB231 cells (n =4). B), Infection of R3616 in normoxic and hypoxic (5% O2) MDA-MB231 cells (n=1). C), Infection of R3616 in normoxic and hypoxic MCF-7 cells (n=4) (2%O2) and D), Infection of R3616 in normoxic and hypoxic (2% O2) Vero cells.

Figure 2. Plaque forming units obtained from samples of infected MDA-MB-231 hypoxic (A) and normoxic (B) cells.

A. Plaque assay from a sample from hypoxic cells. Sample was serially diluted proceeding from the upper left (10^{-1}) to the lower right (10^{-6})



B. Plaque assay from a sample from normoxic cells. Sample was serially diluted proceeding from the upper left (10^{-1}) to the lower right (10^{-6})

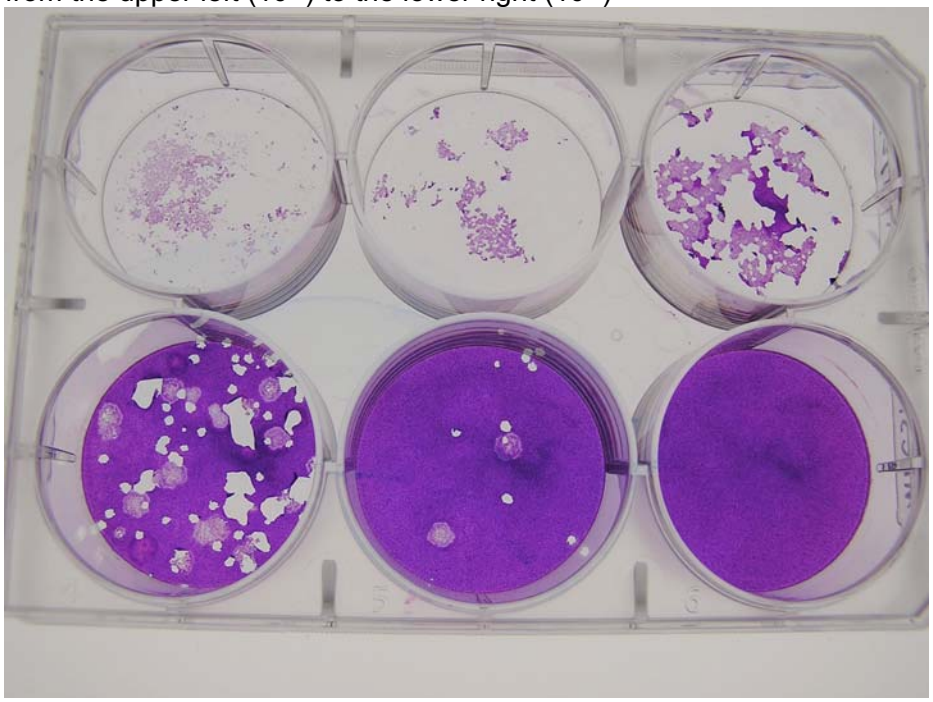
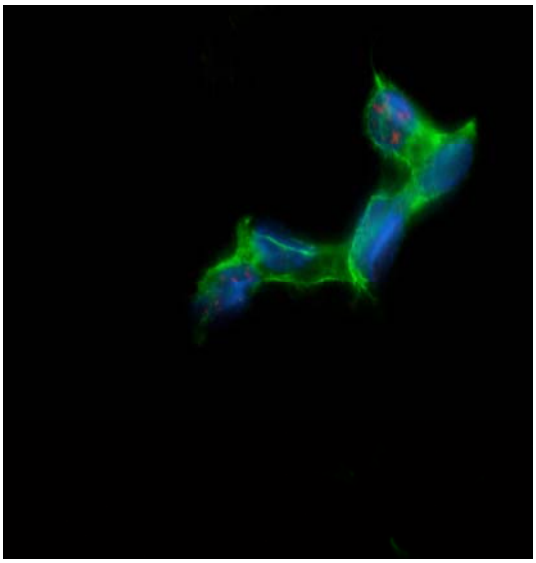


Figure 3. Identification of HSV-1 GP6 (coat protein) in infected MDA-MB-231 normoxic and hypoxic cells by imunofluorescence. Blue staining is the nucleus, red staining is HSC7, and green staining is GP6. A). Normoxic MDA-MB-231 cells after 24 hr. infection with R3616 (M.O.I) = 10. B) Hypoxic MDA-MB-231 cells after 24 hr. infection with R3616 (M.O.I) = 10. C) Normoxic Vero cells after 24 hr. infection with R3616 (M.O.I) = 10

A.



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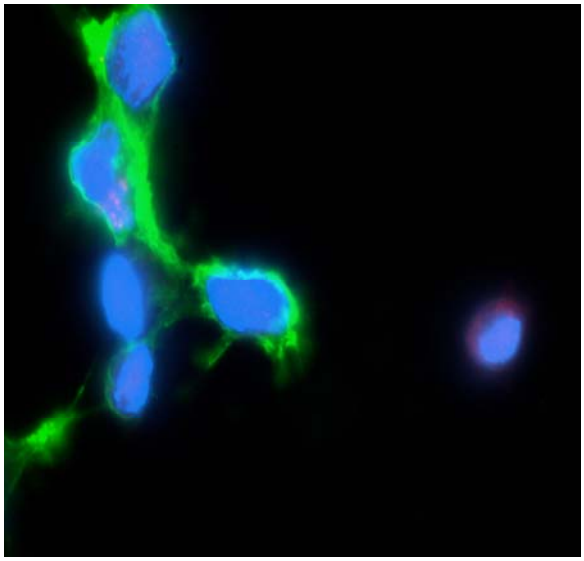

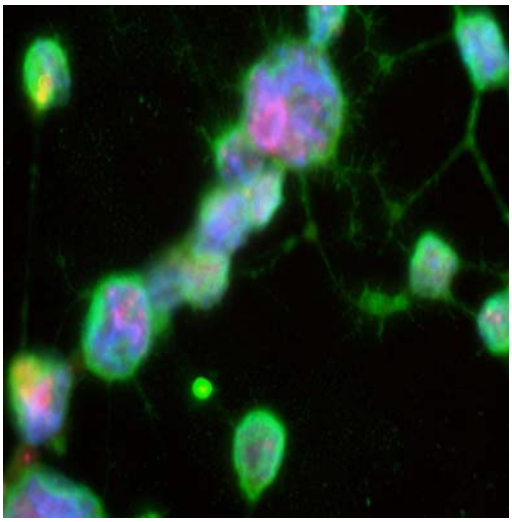

C.